The interplay between ROS and tubulin cytoskeleton in plants

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Plants have to deal with reactive oxygen species (ROS) production, since it could potentially cause severe damages to different cellular components. On the other hand, ROS functioning as important second messengers are implicated in various developmental processes and are transiently produced during biotic or abiotic stresses. Furthermore, the microtubules (MTs) play a primary role in plant development and appear as potent players in sensing stressful situations and in the subsequent cellular responses. Emerging evidence suggests that ROS affect MTs in multiple ways. The cellular redox status seems to be tightly coupled with MTs. ROS signals regulate the organization of tubulin cytoskeleton and induce tubulin modifications. This review aims at summarizing the signaling mechanisms and the key operators orchestrating the crosstalk between ROS and tubulin cytoskeleton in plant cells. The contribution of several molecules, including microtubule associated proteins, oxidases, kinases, phospholipases, and transcription factors, is highlighted.

Reactive oxygen species (ROS) appeared as a consequence of the introduction of oxygen into earth's atmosphere.[1] ROS collectively comprise a group of radical derivatives of oxygen including superoxide anion ($\cdot O_2^-$) and hydroxyl radicals ($\cdot OH$), or non radicals such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2).[2] They are extremely reactive molecules that display different properties from molecular oxygen or other chemical species and are capable of inducing severe damages in cellular components. However, due to their unique chemical features, ROS have evolved as essential regulators in various biological processes.[1,3] In animal cells, they are produced as byproducts of oxygen metabolism or after enzymatic activity in mitochondria, cytoplasm and extracellular sites.[4] In plants, they are generated accidentally or even deliberately in fluctuations in various cellular compartments, such as mitochondria, chloroplasts, peroxisomes as well as the apoplast.[3] ROS are critical for pollen tube growth, gametogenesis and embryo development, root hair development, and stomatal function,[5-8] and they mediate plant responses to hormonal stimuli.[9] Besides their levels rapidly increase during stress resulting in the so-called oxidative burst.[10] Hence, ROS generation is of particular importance for the interaction of plants with biotic factors,[11] and during abiotic stresses including salt, cold, osmotic, and drought stress.[8,10] The crucial contribution of ROS in a broad range of plant developmental and stress processes lies on their ability to act as signals, carry messages, and trigger transduction mechanisms.[12]

On the other hand, microtubules (MTs) are fundamental organelles of plant cells intimately involved in regulation of their morphogenesis[13] and undergo extensive reorganization during abiotic stress[14] or biotic interactions.[15] Increasing evidence indicates that ROS and MTs are team players and that their fates converge during plant life.[16,17] Furthermore, ROS have been repeatedly shown to influence tubulin cytoskeleton in various ways.[18] This review attempts to summarize the existing information relevant to ROS and MTs in plants, trying to shed some light on their emerging crosstalk.

ROS homeostasis and tubulin cytoskeleton during plant life

ROS oscillations occur along with fluctuations of antioxidants throughout plant cell division.[19,20] Apart from its role in regulating specific cell cycle checkpoints,[19] ROS homeostasis, namely the balance between ROS generation and scavenging,[1,10] is critical for proper completion of cell division due to its interference with mechanisms that direct the organization of successive MT arrays in dividing cells.[17,18] The chemically-induced disturbance of ROS homeostasis, assessed by using selective ROS-sensitive fluorescent probes, severely affects the MT arrays. Oxidative stress induced by hexavalent chromium[21] or other chemicals[17,18] disrupts mitosis and cytokinesis in root-tip cells of angiosperms. The elimination of cellular ROS levels results in almost the same effects. Diphenylene iodonium, a specific inhibitor of ROS-producing NADPH oxidase and the ROS scavenger n-acetyl-cysteine harms mitotic and cytokinetic MT arrays in *Triticum turgidum* and *Arabidopsis thaliana* root tips.[18] What's more, many cells of *rhd2 A. thaliana* mutant lacking the function of a NADPH oxidase display low ROS levels in roots and simultaneously defects on MT systems.[17,18] In addition, exogenous application of the antioxidant tripeptide glutathione on *Picea abies* cells affects interphase cortical MTs and the MT-preprophase band.[22] The effects of ROS imbalance on MTs are not restricted
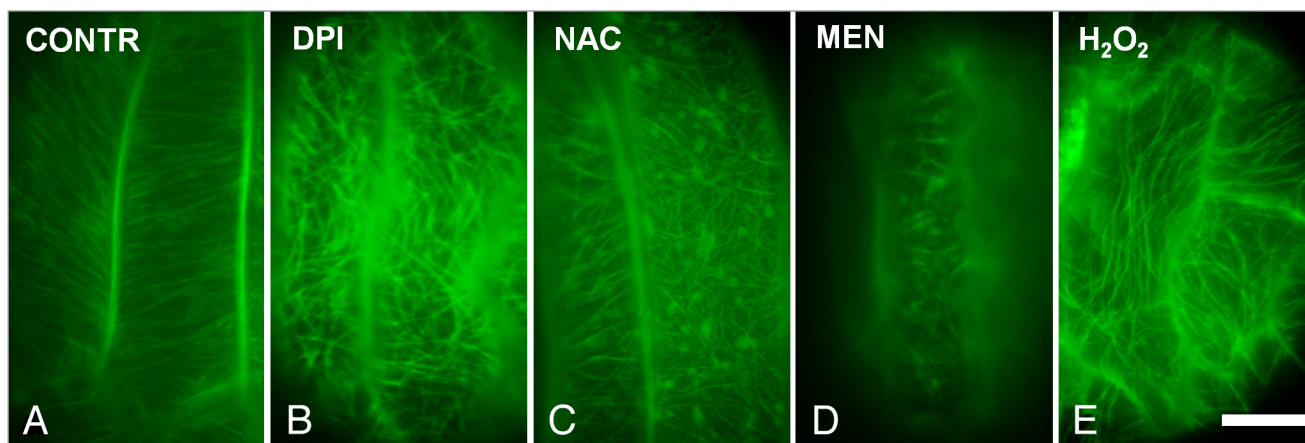
Figure 1. The disturbance of reactive oxygen species (ROS) homeostasis affects tubulin cytoskeleton organization in transgenic *Arabidopsis* plants expressing GFP:TUA6. (**A-E**): Epidermal cells from 4-d-old hypocotyls of seedlings treated with distilled water (**A**) or with ROS modulators (**B-E**). The decrease of ROS levels forced either by the NADPH oxidase inhibitor diphenylene iodonium (DPI) or by the ROS scavenger n-acetyl-cysteine (NAC) results in disruption and reorganization of the tubulin cytoskeleton into random mesh-like arrays (**B, C**; cf. **A**). Similarly, oxidative stress induced by the oxidizing agent menadione (MEN) and hydrogen peroxide (H_2O_2) results in MT remodeling (**D, E**; cf. **A**). CONTR, distilled water; DPI, 50 μM; NAC, 500 μM; MEN, 50 μM, H_2O_2, 5 mM. Treatments: 2 h. Scale bar: 10 μm.

to dividing cells. Treatments of GFP:TUA6 transgenic *Arabidopsis* seedlings with ROS modulators revealed that the cortical MTs in developing epidermal cells of hypocotyls and cotyledons are also sensitive to alterations in ROS levels (**Fig. 1**). Moreover, *Arabidopsis* epidermal cells exposure to ROS-generating titanium dioxide (TiO_2) nanoparticles disrupted the MT networks and led to formation of tubulin aggregates.[23] Hence, either ROS overproduction or elimination forces the disappearance of MTs and their replacement by stable and resistant atypical tubulin polymers (see also ref. 24). These polymers may be macrotubules i.e., tubulin tubules displaying higher outer diameter compared with typical MTs, or paracrystal conformations containing tubulin referred as tubulin paracrystals.[18]

On the other hand, elevation of ROS levels has been recorded during almost every type of biotic or abiotic stress and functions as a universal signal triggering downstream responses.[25] Stress-induced ROS generation occurs in different cellular sources, activates mitogen-activated protein kinase (MAPK) cascades and leads to upregulation of several transcription factors.[26] In addition, many different kinds of stress induce rearrangements in tubulin cytoskeleton.[14,15] MTs participate in biotic stress responses, including the pathogen attack-induced programmed cell death.[27] Likewise, disruption and depolymerization of MTs at the site of pathogen infection has been repeatedly observed.[15,27] Moreover, MT reorganization occurs along with oxidative burst during mechanical stress and both these phenomena are implicated in sensing mechanical stimulation.[28,29] Therefore, it is reasonable to assume that ROS generation and cytoskeletal alterations might be coupled during plant life. Both drive developmental procedures and, during stress, they promote the perception of the stimuli, as well as the consequent responses.

Implications of cytoskeletal contribution to deliberate ROS production

One of the most important protein groups that respond to stress and concurrently contribute to ROS production is that of Rbohs

(respiratory burst oxidase homologs).[10] They are plasmalemmal enzymes that catalyze superoxide production in the apoplast.[30] Superoxide may be protonated to form hydroperoxyl radical ($\cdot O_2H$) or converted to hydrogen peroxide, which easily diffuses into the cytosol and initiates signaling transduction.[30] The activation of NAPDH oxidases followed by ROS generation has been recorded in defense responses of *A. thaliana* against *Verticillum dahliae* toxins[31] or those against the bacterial elicitors flg22 and Harpin in *Vitis* cells.[32] Using diphenylene iodonium alone or together with exogenously added hydrogen peroxide Yao et al.[31] showed that the NADPH oxidase-mediated ROS accumulation is responsible for MT-depolymerization. In turn, the depolymerization of cortical MTs has been repeatedly suggested to be essential for the expression of specific resistant genes.[31,32]

Recent evidence suggested the existence of a positive feedback relationship between tubulin cytoskeleton and ROS production and revealed a new mode of NADPH oxidase regulation. In this relationship, the MT-associated protein MAP65-1 displays a unique role, different than usual. ZmMAP65-1a that was found to be increased after application of hydrogen peroxide was also shown to regulate hydrogen peroxide amplification upon treatment of *Zea mays* leaves with brassinosteroid phytohormones.[33] The ROS-responsive ZmMPK5 interacts with ZmMAP65-1a toward activating the expression of NADPH oxidase genes and the subsequent responses.[33] In particular, the transcription of several antioxidant defense genes, which is initiated after NADPH oxidase activation, requires the phosphorylation of ZmMAP65-1a by ZmMPK5.[33,34] Therefore, it is very interesting that, during stress, cytoskeletal proteins respond to elevated ROS levels and at the same time activate the NADPH oxidase assisting the purposeful production of ROS.

The implication of NADPH oxidase and ROS homeostasis in regulation of MT organization is further supported by the case of *rhd2 A. thaliana* mutants, which lack one of the *A. thaliana* NADPH oxidases. The *rhd2* mutants display low ROS levels and simultaneously tubulin cytoskeleton defects and a significant

amount of macrotubules in roots.[18] Interestingly, these plants exhibit extremely elevated levels of a p46 p38-like MAPK.[35] It was recently found that in angiosperms the p46 MAPK is implicated in sensing cellular ROS level alterations. ROS overproduction as well as elimination of ROS levels resulted in the activation of this protein.[35] Once activated, p46 drives the assembly of atypical tubulin polymers.[35] This strengthens the view that the mechanisms maintaining homeostasis during low or elevated ROS levels are connected by feedback loops and tightly linked to cytoskeletal responses.

ROS signaling and the role of phosphatidic acid

The p46 MAPK is assumed to be directly or indirectly involved in MAP65-1 activity toward the assembly of atypical tubulin polymers during ROS imbalance.[35] Moreover, MAP65-1 is phosphorylated by several other ROS-responsive MAPKs. AtMPK3, AtMPK4, and AtMPK6, which are activated by ROS,[36] have been shown to directly phosphorylate AtMAP65-1.[37,38] The activity of MAP65-1 is also modulated by its interaction with phosphatidic acid (PA), an important lipid mediator produced by phospholipase D (PLD).[39] PA and MAP65-1 synergy plays a critical role during salt stress, since PA binds to MAP65-1 and enhances tubulin polymerization and the MT-bundling activity of MAP65-1.[39] Salinity forces MT depolymerization followed by MT reorganization into stable arrays, responses leading to salt tolerance. The oxidative burst seems to have a significant part in these processes.[24] PA production and activation of p46 p38-like MAPK mediate the assembly of macrotubules during protoplast volume regulation in plasmolysed *T. turgidum* root-tip cells.[40] In *A. thaliana*, PA produced by PLD regulates ROS generation by NADPH oxidase during stomatal closure induced by treatment with abscisic acid.[41] Interestingly, in *Vicia* a p38-like MAPK participates in abscisic acid triggered oxidative burst, whereas this hormone was found to activate p38 MAPK in chloronema cells of *Funaria hygrometrica*.[42,43] Considering the data presented above, it may be proposed that the interactions between PLD and its derivative PA, p38-like MAPKs and NAPDH oxidase-derived ROS collectively orchestrate the rearrangements of tubulin cytoskeleton, which are part of the responses to external stimuli.

This implies that ROS mediate stress responses and hormone signaling, through converging pathways. Such responses repeatedly include participation of the tubulin cytoskeleton and recruitment of tubulin associated proteins. In this direction, ZmMPK5 is activated by abscisic acid[44] and concurrently, as it was previously noticed, interacts with ZmMAP65-1a toward ROS production after treatment with brassinosteroids.[33] Abscisic acid also strongly modifies the levels of α and β tubulin mRNA transcripts[45] and induces depolymerization and reorganization of cortical MTs.[46,47] In addition, it is known that the rest classes of phytohormones also modulate ROS levels in plants,[9] whereas almost all the hormones interfere with plant cytoskeleton.[48,49] Although, some implications suggest ROS involvement in hormone- induced tubulin cytoskeleton remodeling, their exact role cannot be easily identified.

MT-associated transcription factors involved in fine tuning of ROS levels

An attractive example of a possible interconnection between MTs, hormones and effector molecules are DELLA proteins. These proteins are transcription factors, which participate in hormone signaling mechanisms[50] and mediate several developmental processes in plants.[51] During stress, DELLA proteins promote the transcription of genes encoding antioxidant proteins, such as superoxide dismutase. Hence, they contribute to fine tuning of cellular ROS levels.[50,52] Importantly, DELLA proteins have been recently implicated in governing cortical MT organization in *Arabidopsis* during cell expansion induced by gibberellins.[51] In particular, these proteins affect, among others, the availability of α/β tubulin heterodimers and consequently the polymerization of MTs due to their ability to direct the prefoldin complex localization, which is required for tubulin folding. Furthermore, Locascio et al.[51] note that the DELLA-dependent accumulation of prefoldin complex in nucleus is potentially crucial for the expression of several tubulin encoding genes. This is very interesting since it is known that the disturbance of ROS homeostasis affects the levels of α or β tubulin in plants and animals, leading in some cases to elevated tubulin levels (see also ref. 23).[17,53] We may then extrapolate that the upregulation of tubulin is part of the antioxidant defense responses induced by transcriptional mediators like DELLA proteins. In this direction, tubulin upregulation may follow treatments with chemicals inducing low ROS levels.[17] These data further support the hypothesis that the dynamic regulation of tubulin cytoskeleton is part of the cellular mechanisms driving the restoration of ROS homeostasis.

Some aspects on the oxidation of tubulins

Paradigms from animal cells support the view that the regulation of tubulin cytoskeleton act protectively against pertubations of cellular redox state.[54] It is well known that tubulins are extremely sensitive to oxidative stress and that their cysteine residues could be easily oxidized, even after a short exposure to ROS.[54] Oxidation of cysteine sulfhydryl groups may promote the cross-linking between α and β tubulin due to formation of disulfide bonds, thus preventing the assembly of MTs.[54,55] Of outstanding interest is the case of βIII isoform of animal β tubulins (reviewed by Ludueña[54]). This isoform lacks cys239, one of the cysteines of β tubulin, which easily forms disulfide bonds and its oxidation has been straightly correlated with the inhibition of MT assembly.[54] Importantly, the βIII isoform is present at high levels in cells displaying regularly elevated ROS levels and is upregulated in oxidative stress treated cells.[54] Therefore, it seems that redox sensing may be related to the expression of different tubulin isoforms and this may prevent cellular damage.

Such information regarding plant cell tubulins is missing. However, proteome examination of *A. thaliana* cells treated with hydrogen peroxide revealed that α and β plant tubulins are also susceptible to oxidation.[56] Besides, using proteomic analysis it was found that both α and β tubulins undergo S-glutathionylation in *A. thaliana* cells, after application of the oxidant *t*-butyl-hydroperoxide (*t*-BuOOH).[57] Glutathione binding offers protection to thiol groups of cysteine against oxidation. Nevertheless, thiolation itself could influence the activity of the modified protein or even drive signal transduction.[57,58] Thus, it seems that apart from mechanisms that perceive cellular redox status, additional regulatory mechanisms sense the redox status of tubulin. Since the later is strongly related to the
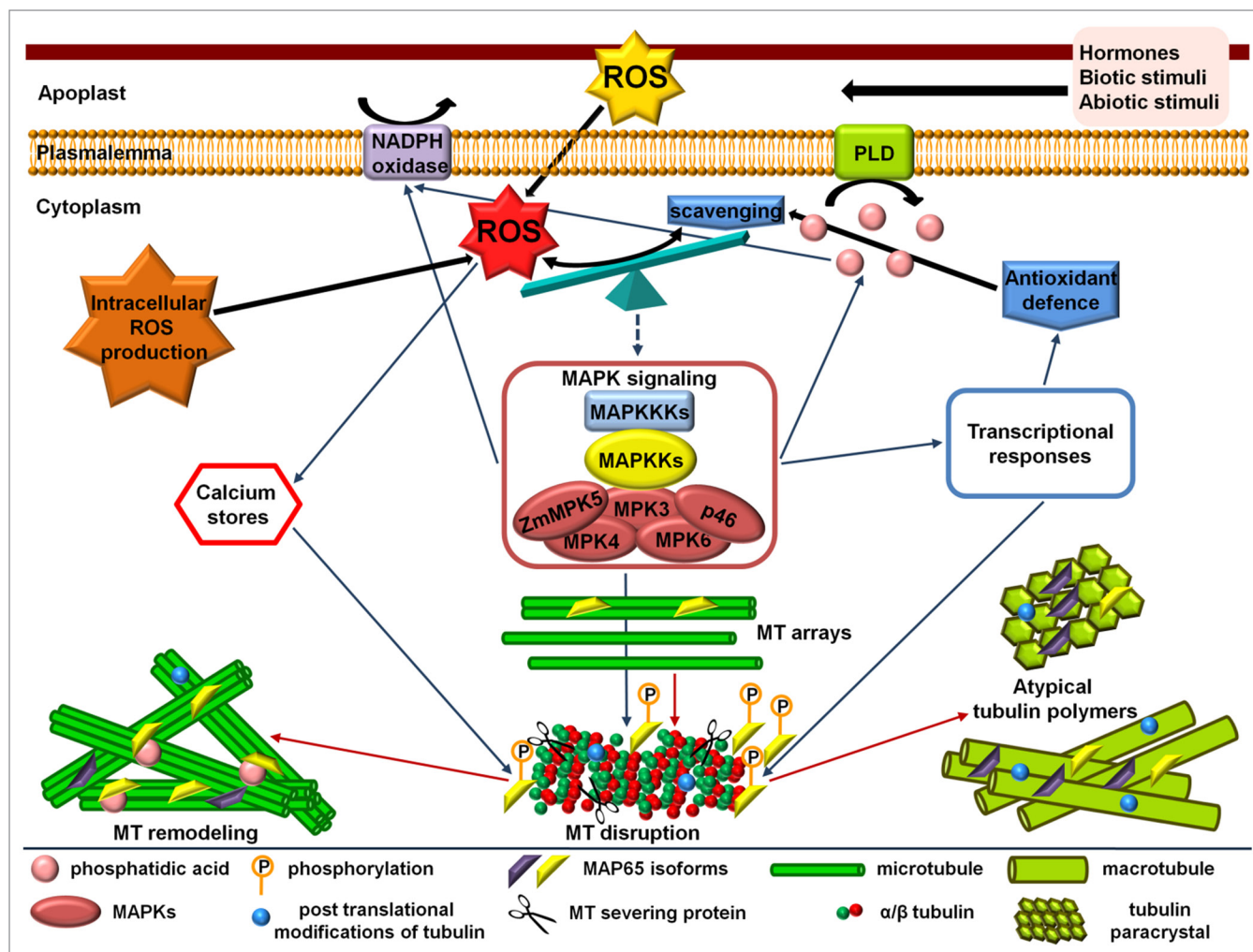
Figure 2. Simplified hypothetical model describing the changes mediated by reactive oxygen species (ROS) level alteration with respect to the organization of tubulin cytoskeleton. ROS and antioxidant balance is further influenced by abiotic or biotic stimuli. In any case, the ROS produced in the apoplast (e.g., derived from NADPH oxidase) enter the cytosol and together with intracellularly generated ROS constitute the total amount of cellular ROS. Consequently, the result of the tag war between oxidant generation and antioxidant defenses is perceived and either directly or through feedback connected signaling cascades forces the reorganization of tubulin cytoskeleton. The reorganization among others aids the restoration of ROS homeostasis. The microtubule (MT) arrays disappear and MT remodeling, or the assembly of atypical tubulin polymers like macrotubules and tubulin paracrystals, follows. These processes are mediated by MT-associated proteins (MAPs) assisted by other molecules such as phosphatidic acid produced by phospholipase D (PLD). The modifications of tubulin or MAPs at the post-translational level may crucially contribute to this interplay.

capability for MT assembly,[59,60] such mechanisms may protect tubulin against irreversible oxidation.[60]

Tubulin cytoskeleton remodeling during ROS imbalance

The formation of stable atypical tubulin polymers, following the disturbance of ROS homeostasis, can be explained considering that they contain or consist of modified tubulins. Apart from oxidative modifications ROS imbalance was found to rapidly induce other post-translational modifications in plants, such as tubulin acetylation[17,18] (see also ref. 21) or slight alterations of the tyrosinated tubulin levels.[17] Such modifications that amend the tubulin polymer properties are common in stress conditions.[32] For example, hyperosmotic treatment stimulates phosphorylation of α-tubulin.[61] Interestingly, osmotic stress results not only in cellular oxidative burst,[10] but also in disappearance of MTs followed by the assembly of macrotubules.[40] ROS production and its interplay with

MT reorganization allow adaptive response during osmotic stress in a model proposed by Nick et al.[62] Taking into consideration the data presented above, we may propose that tubulin post-translational modifications during ROS homeostasis disturbance could be correlated with the behavior of the tubulin cytoskeleton. A possible explanation of this behavior might be that such modifications probably, among others, influence the interaction of tubulins or MTs with associated macromolecules (see also ref. 63) as well as the properties of tubulin polymers.

Regarding the process of MT destruction, one would suggest that ROS might directly or indirectly interfere with aspects of proper tubulin polymerization, such as the concentration of calcium or the availability of free tubulin heterodimers.[64] It is known that increased ROS levels are implicated in raising calcium levels[7] and that MTs are sensitive to calcium concentrations.[64] Elevated

calcium may lead to destruction of MTs[65] and ROS might interfere with the amount of tubulin available for polymerization.[17] The disruption of MTs could potentially be mediated by the activation of MT severing proteins like katanin. Katanin is a heterodimeric protein that binds to and cleaves MTs.[66] In plants, it was shown to be recruited during cytoskeletal responses to several stimuli that induce elevation of cellular ROS levels, such as mechanical stress and wounding,[8,28,29] blue light,[67,68] and hormonal responses.[9,66] However, the extent of association of ROS fluctuations with katanin activation remains to be found. Besides, MT disappearance could be further promoted by weakening of MAP binding to MTs. For instance, phosphorylation of MAP65–1, which belongs to MAP65 protein family and has been implicated in MT bundling, results in its dissociation from MTs and leads to MT disassembly.[37] Therefore, it is very interesting that MAP65–1 is one of the substrates of the ROS-activated MAPKs, AtMPK3, AtMPK4, AtMPK6.[36-38]

Importantly, MAP65–1 mediates the formation of atypical tubulin polymers, i.e., macrotubules and tubulin paracrystals during ROS disturbance.[18] MAP65 proteins also participate in the assembly of tubulin paracrystals after treatment of *Vigna sinensis* roots with colchicine.[69] What's more, the MAP65–6 along with MAP65–1 has been found to promote the organization of resistant thick MT bundles during salt stress, which appear as a mesh-like network.[70] The binding capacity of different MAP65 proteins to MTs highly depends on the MT-binding domains of MAP65 isotypes, which are localized to the C-terminal of each protein molecule.[71] Besides, post-translational modifications of tubulin could modulate the binding potential to different MAPs.[63] Hence, we may suggest that, during atypical tubulin polymer formation accompanying loss of ROS homeostasis, MAPs promoting the assembly of tubulin polymers other than MTs preferably bind to modified tubulin.

Concluding Remarks

ROS homeostasis is of particular importance for plant life and, nowadays, emerges as a significant regulator of tubulin cytoskeleton. The interplay between ROS and tubulin cytoskeleton is implicated in sensing almost every stressful situation and drives the succeeding responses. The responses against various stimuli are mediated through converging signaling cascades that aim at restoring ROS homeostasis. During this process the remodeling of tubulin cytoskeleton seems to play a crucial role. Such remodeling includes, among others, the recruitment of MAPs which is forced by MAPK signaling and regulatory mechanisms acting at transcriptional and post-transcriptional level. Some of the possible participants of this complex crosstalk, which were discussed in the present article, are illustrated in **Figure 2**. The existence of other signaling routes, protein components and additional mechanisms cannot be excluded. Further experimentation is needed in order to establish a more clear relationship between ROS homeostasis and tubulin cytoskeleton. ROS appear as ideal players in fine tuning of the behavior of tubulin cytoskeleton during stress. It would therefore be very promising to further investigate the ROS-dependent MT-mediated developmental processes.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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